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Heterogeneity in the A33 protein impacts the cross-protective efficacy of a candidate smallpox DNA vaccine

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ABSTRACT

We previously developed a gene-based vaccine, termed 4pox, which targets four orthopoxvirus proteins (A33, L1, B5, and A27). Because any subunit orthopoxvirus vaccine must protect against multiple species of orthopoxviruses, we are interested in understanding the cross-protective potential of our 4pox vaccine target immunogens. In our current studies, we focused on the A33 immunogen. We found one monoclonal antibody against A33, MAb-1G10, which could not bind the monkeypox virus A33 ortholog, A35. MAb-1G10 binding could be rescued if A35 amino acids 118 and 120 were substituted with those from A33. MAb-1G10 has been shown to protect mice from VACV challenge, thus our findings indicated a protective epitope differs among orthopoxviruses. Accordingly, we tested the cross-protective efficacy of a DNA vaccine consisting of A35R against VACV challenge and compared it to vaccination with A33R DNA. Mice vaccinated with A35R had greater mortality and more weight loss compared to those vaccinated with A33R. These findings demonstrate that despite high homology between A33R orthologs, amino acid differences can impact cross-protection. Furthermore, our results caution that adequate cross-protection by any pan-orthopoxvirus subunit vaccine will require not only careful evaluation of cross-protective immunity, but also of targeting of multiple orthopoxvirus immunogens.

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Introduction

In response to the potential threat of variola virus (VARV) or a genetically modified poxvirus being accidentally or purposefully released, there is renewed interest in orthopoxvirus vaccination (Bray and Buller, 2004; Enserink, 2004; Moore et al., 2006). Additionally, monkeypox virus (MPXV) continues to cause morbidity and mortality in areas of Africa (Parker et al., 2007; Rimoin et al., 2007), and the accidental importation of monkeypox-infected animals recently caused a monkeypox outbreak in the Midwestern United States (Reed et al., 2004). The historic smallpox vaccine, Dryvax, is composed of live vaccina virus (VACV) and is based on technology developed over 200 years ago by Edward Jenner. A plaque-purified VACV grown in cell culture, ACAM2000 was recently deemed safe and effective by the Food and Drug Administration (Artenstein et al., 2005; Monath et al., 2004). Despite being highly protective, these live-virus smallpox vaccines can be associated with significant adverse events, including spread of the virus to other sites on the body, including the eye (Lane and Goldstein, 2003), and spread to other people in close contact to the vaccinee. More serious complications can include myocarditis, progressive vaccinia, eczema vaccinatum, and even death (Bray, 2003; Cassimatis et al., 2004; Eckart et al., 2004; Kretzschmar et al., 2006; Wharton et al., 2003; Wollenberg et al., 2003). As a result, this vaccine is contraindicated in large segments of the population, including the immune compromised and individuals with various dermatological conditions, such as atopic dermatitis (Bray, 2003). Attenuated versions of the live-virus vaccine have been generated (e.g., MVA and LC16m8); however, these attenuated viruses still contain hundreds of genes, many of which encode for immunomodulatory molecules or molecules with unknown function. The safety risks posed by these molecules remain unexplored. In response to these negative aspects of live-orthopoxvirus vaccines, subunit vaccines consisting of orthopoxvirus genes and/or proteins are currently under development (Fang et al., 2006; Fogg et al., 2004, 2007; Heraud et al., 2006; Hooper et al., 2000, 2003, 2007, 2004; Sakhatskyy et al., 2006, 2008; Xiao et al., 2007).

Our laboratory has developed a candidate gene-based vaccine, termed 4pox, which targets four orthopoxvirus proteins (A33, L1, B5 and A27). The A33R, L1R, B5R, and A27L genes encode the four proteins, A33, L1, B5, and A27. All of these immunogens are highly homologous (>93%) between VACV, MPXV, and VARV. This vaccine targets both infectious forms of orthopoxviruses, the mature virion (MV) (L1R and A27L), and the enveloped virion (EV) (A33R and B5R). MVs and EVs are antigenically distinct from each other and interact with the cell surface differently (Moss, 2006; Smith and Vanderplasschen, 1998; Smith et al., 2002; Vanderplasschen and Smith, 1997). MVs are believed to be involved in host-to-host spread, while EVs are thought to be involved in intra-host viral dissemination. By targeting both particle types, this vaccine is designed to reduce input virus (MV), quench intra-host

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14. ABSTRACT
We previously developed a gene-based vaccine, termed 4pox, which targets four orthopoxvirus proteins (A33, L1, B5, and A27). Because any subunit orthopoxvirus vaccine must protect against multiple species of orthopoxviruses, we are interested in understanding the cross-protective potential of our 4pox vaccine target immunogens. In our current studies, we focused on the A33 immunogen. We found one monoclonal antibody against A33, MAb-1G10, which could not bind the monkeypox virus A33 ortholog, A35. MAb-1G10 binding could be rescued if A35 amino acids 118 and 120 were substituted with those from A33. MAb-1G10 has been shown to protect mice from VACV challenge, thus our findings indicated a protective epitope differs among orthopoxviruses. Accordingly, we tested the cross-protective efficacy of a DNA vaccine consisting of A35R against VACV challenge and compared it to vaccination with A33R DNA. Mice vaccinated with A35R had greater mortality and more weight loss compared to those vaccinated with A33R. These findings demonstrate that despite high homology between A33R orthologs, amino acid differences can impact cross-protection. Furthermore, our results caution that adequate cross-protection by any pan-orthopoxvirus subunit vaccine will require not only careful evaluation of cross-protective immunity, but also of targeting of multiple orthopoxvirus immunogens.

15. SUBJECT TERMS
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spread (EV) and also limit viral shedding. Previously, we showed that the 4pox vaccine using VACV or MPXV genes is capable of protecting mice and non-human primates (NHP) from lethal challenge with VACV or MPXV (Heraud et al., 2006; Hooper et al., 2000, 2003, 2007, 2004 and J.W. Hooper, unpublished results). Others further confirmed the protective efficacy of these vaccine target combinations using recombinant protein (Fogg et al., 2004; Xiao et al., 2007). However, it remains unclear how well these vaccine immunogens individually contribute to protection against a heterologous viral challenge. Because any subunit orthopoxvirus vaccine must protect against multiple species of orthopoxviruses (e.g., VACV, MPXV, and VARV), we are interested in understanding the cross-protective potential of our 4pox vaccine target immunogens. To begin to investigate cross-protection, we initially focused on the A33R component of the 4pox vaccine.

The VACV A33R open reading frame encodes the A33 protein. A33 is a type II integral membrane protein present as a dimer on the EV (Roper et al., 1996; Smith and Vanderplasschen, 1998). Some evidence suggests A33 has a role in facilitating antibody-resistant cell-to-cell spread of orthopoxviruses (Law et al., 2002). It has also been shown to interact with other orthopoxvirus proteins, A36R and B5R (Wolffe et al., 2001; Perdiguero and Blasco, 2006). Although non-essential for replication (Roper et al., 1998), antibodies against A33 are protective *in vivo* (Galmiche et al., 1999; Hooper et al., 2002; Lustig et al., 2005; Chen et al., 2007). For example, monoclonal antibody (MAb)-1G10 is capable of passively protecting animals from lethal challenge with VACV strain WR (Lustig et al., 2005). In addition to being a target of immunotherapeutics, A33 was identified as a protective target of subunit vaccines delivered as DNA or protein, alone or in combination with other protective immunogens (Fogg et al., 2004, 2007; Galmiche et al., 1999; Heraud et al., 2006; Hooper et al., 2000, 2003, 2007, 2004; Sakhatskyy et al., 2008; Xiao et al., 2007). Despite representing a critical target for therapeutic intervention, the regions of A33 critical for antibody-mediated protection are unknown. The mechanism by which antibodies mediate protection is also unclear. Here, we explored a finding that an anti-VACV A33 MAb was unable to efficiently cross-react with the MPXV A33 ortholog, A35. This observation allowed us to identify amino acids associated with a protective antibody epitope on the A33 ortholog. Because a protective epitope differed among VARV, MPXV and VACV, we also examined the effect that heterogeneity in the A33 molecule had on the cross-protective efficacy of an A33 ortholog-based DNA vaccine against orthopoxvirus challenge. Throughout this study we refer to the A33R ortholog genes as $A33R^{VACV}$ (A33R), $A33Ro^{MPXV}$ (A35R), and $A33Ro^{VARV}$ (A36R) and the encoded proteins as $A33^{VACV}$ (A33), $A33o^{MPXV}$ (A35), and $A33o^{VARV}$ (A36), where "o" designates ortholog.

Results

Interaction of anti-$A33^{VACV}$ MAbs with $A33o^{MPXV}$

We first became interested in the potential impact of A33R heterogeneity on protective immunity after observing that one of our protective anti-A33 mouse MAbs failed to bind $A33o^{MPXV}$. In that experiment, two MAbs against $A33^{VACV}$, MAb-10F10 and MAb-1G10, were tested for binding to $A33o^{MPXV}$ by radiolabeled immunoprecipitation analysis (RIPA). As expected, both MAb-1G10 and MAb-10F10 immunoprecipitated $A33^{VACV}$; however, MAb-10F10, but not MAb-1G10, immunoprecipitated $A33o^{MPXV}$ (Fig. 1). To confirm the RIPA data, interaction of MAb-10F10, MAb-1G10, and a control antibody MAb-10F5 (anti-L1), was tested for binding to purified $A33^{VACV}$ and $A33o^{MPXV}$ by ELISA. Both MAb-1G10 and MAb-10F10 bound to purified $A33^{VACV}$; but only MAb-10F10 was capable of binding to purified $A33o^{MPXV}$, even at the lowest dilution tested (Fig. 1B). The control antibody against orthopoxvirus L1 (MAb-10F5) did not interact with either A33 ortholog. These findings demonstrated that protective antibodies to at least one $A33^{VACV}$ epitope poorly bind $A33o^{MPXV}$.

Reduction of A33 disrupts the epitope bound by MAb-10F10 and MAb-1G10

Antibody epitopes can be either linear or dependent upon tertiary structure (i.e. conformational). To gain insight as to the nature of the MAb-10F10 and MAb-1G10 antibody epitopes, we investigated the effect reducing $A33^{VACV}$ had on antibody interactions. To this end, lysates of COS cells transfected with $A33R^{VACV}$ or empty vector were either unreduced or reduced with β-mercaptoethanol and subject to immunoblotting using MAb-10F10 and MAb-1G10. Both MAb-10F10 and MAb-1G10 interacted with A33 under non-reducing conditions. In sharp contrast, neither antibody interacted with A33 when the molecule was reduced (Fig. 1C). These findings strongly suggested that epitopes bound by MAb-10F10 and MAb-1G10 are conformationally dependent.

Interaction of anti-$A33^{VACV}$ MAbs with VACV and MPXV-infected cells

Because the epitopes bound by both MAb-1G10 and MAb-10F10 were conformationally dependent (Fig. 1C), we reasoned that while MAb-1G10 did not bind recombinant $A33o^{MPXV}$ as determined by ELISA and RIPA, it was possible that this antibody could bind to the authentic protein expressed in cells infected with MPXV. This could be due to conformational changes induced when the molecule is localized in a viral or cellular membrane or due to interaction(s) with other viral protein(s), such as an ortholog of the $A36^{VACV}$ or $B5^{VACV}$ molecules (Rottger et al., 1999; Smith et al., 2002; Wolffe et al., 2001; Perdiguero and Blasco, 2006). Therefore, we examined the capacity of MAb-10F10 and MAb-1G10 to interact with A33o in cells infected with VACV, strain IHD-J, or MPXV, strain Zaire79. VACV strain IHD-J was chosen because this is the strain we typically use to perform challenge studies (see below). The A33R from VACV strain IHD-J and strain Connaught are highly homologous. There are two nucleotide (nt) changes (A to G at position 276 and G to A at 511). The nt change at 276 is silent and the nt change at 511 results in a V to I substitution at amino acid position 171 (data not shown). COS cells were infected with IHD-J or MPXV and 48-h post-infection cells were fixed and stained with MAb-10F10, MAb-1G10 or a control antibody MAb-3D7 (anti-Hantaan G2), followed by a secondary anti-mouse antibody conjugated to Alexfluor488. MAb-10F10 bound to both VACV- and MPXV-infected cells (Fig. 1D). In contrast, MAb-1G10 bound VACV-infected cells, but failed to efficiently bind MPXV-infected cells. The reactivity that was observed with MAb-1G10 was markedly lower compared to MAb-10F10 and almost below the level of detection (Fig. 1D). Thus, MAb-1G10 bound poorly to $A33o^{MPXV}$ in assays involving not only recombinant protein, but also authentic protein in the context of MPXV-infected cells.

Rescue of MAb-1G10 interaction with $A33o^{MPXV}$

Based on the predicted amino acid sequence, there are eight amino acid substitutions between $A33^{VACV}$ and $A33o^{MPXV}$. The $A33o^{MPXV}$ is also truncated by four amino acids at the carboxy terminus (Fig. 2A). To determine which amino acid difference(s) disrupted the epitope bound by MAb-1G10, we initially examined the role of the amino acid difference at the carboxy terminus of the $A33o^{MPXV}$. A construct was generated that encoded $A33o^{MPXV}$ where the carboxy terminal threonine was replaced with the last five amino acids of $A33^{VACV}$. This construct was designated $A33Ro^{MP/VACV\text{-}Cterm}$. COS cells were then transfected with either $A33Ro^{MP/VACV\text{-}Cterm}$ or $A33R^{VACV}$ and then subjected to RIPA analysis. As shown in Fig. 2B, protein expressed from the $A33Ro^{MP/VACV\text{-}Cterm}$ construct was able to interact with the MAb-10F10 and hyperimmune mouse serum; however, it was not bound by MAb-1G10. These data suggested that the C-terminal amino acid truncation was not involved in MAb-1G10 binding. We next examined the role of residues 117, 118, and 120 in MAb-1G10 binding. All three amino acids

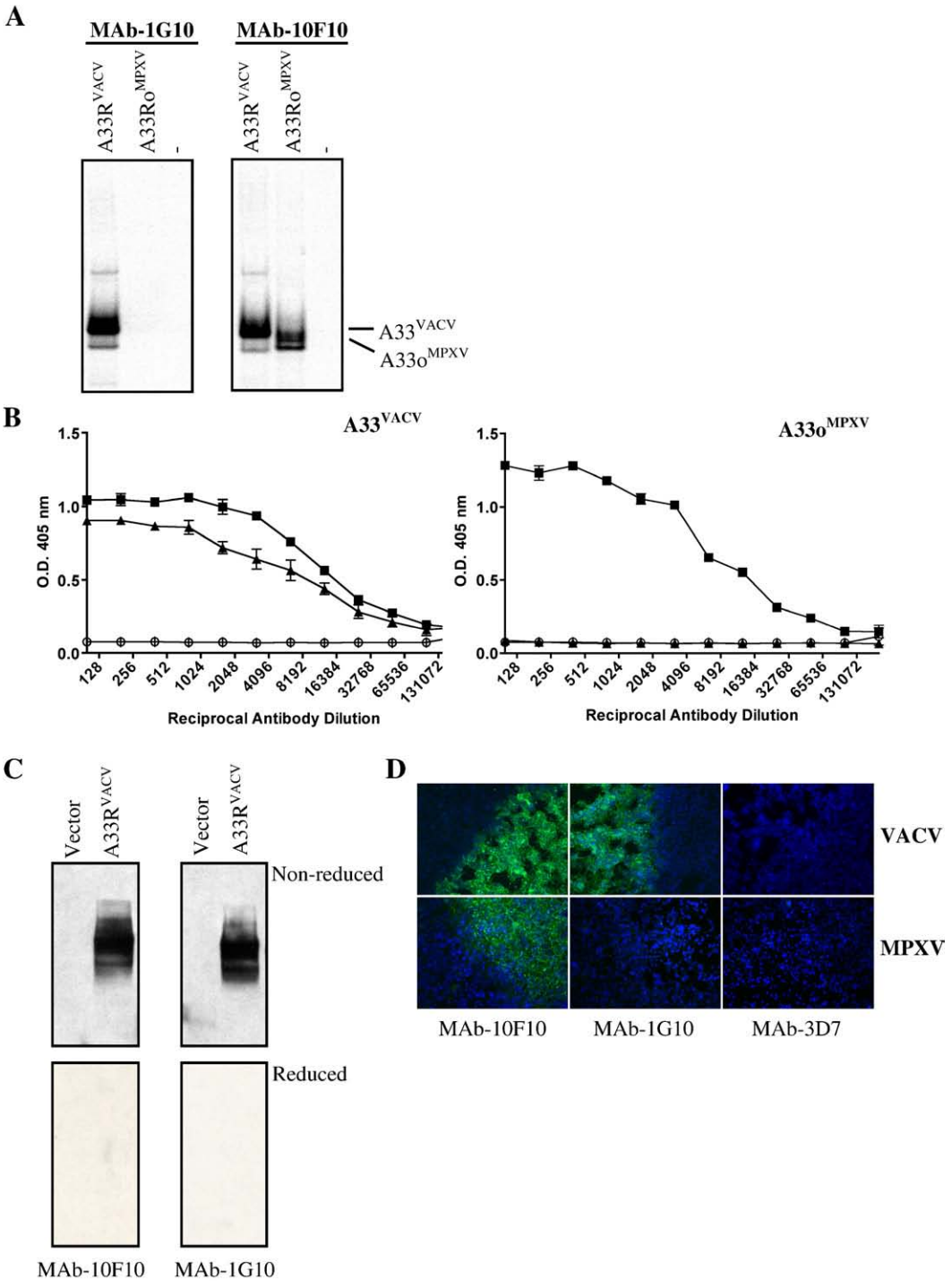

Fig. 1. Interaction of MAbs MAb-1G10 and MAb-10F10 with A33^{VACV} or A33o^{MPXV}. A. COS cells were either not transfected (−) or transfected with pWRG/A33R or pMPXV/A33Ro and labeled with ^{35}S-methionine/cysteine. Cell lysates were then incubated with MAb-10F10 or MAb-1G10. Protein–antibody complexes were precipitated, revolved by SDS-PAGE and subjected to phosphoimaging analysis. B. ELISAs were performed using purified A33^{VACV} or A33o^{MPXV} proteins. Serial dilutions of MAb-10F10 (closed squares), MAb-1G10 (closed triangles) or a control antibody MAb-10F5 (anti-L1) (open circles) were incubated with both proteins then incubated with an anti-mouse secondary antibody conjugated to horseradish peroxidase. Each point represents an average of two samples±standard deviation. C. COS cells were transfected with pWRG/A33R or empty vector. Cells lysates were either untreated or reduced with β-mercaptoethanol, resolved by SDS-PAGE, and transferred to nitrocellulose. Samples were then immunoblotted with MAb-10F10 or MAb-1G10 followed by a secondary anti-mouse IgG conjugated to horseradish peroxidase. D. COS cells were infected with either VACV, strain IHD-J, or MPXV, strain Z79, for 48 h and fixed. Fixed cells were incubated with MAb-10F10, MAb-1G10, or MAb-3D7 (anti-Hantaan G2). Cells were then incubated with a secondary anti-mouse antibody conjugated to Alexfluro488. Coverslips were mounted onto glass slides in mounting medium containing DAPI nucleic acid stain and the edges of plaques were imaged on a fluorescence microscope.

at these positions were mutated into those encoded by the A33^{VACV}, forming a construct designated A33Ro$^{MP117–120}$. COS cells were then transfected with plasmids expressing mutant A33Ro$^{MP117–120}$ or A33R^{VACV} and subjected to RIPA analysis. As shown in Fig. 2C, protein expressed from the mutated A33Ro^{MPXV} construct could interact with MAb-1G10. These data indicated that amino acids 117, 118, and 120 were involved in the epitope bound by MAb-1G10.

We next investigated the individual contribution of each of the three amino acids to MAb-1G10 binding. A33Ro^{MPXV} constructs were generated containing single changes (K117Q, S118L, and E120S) and

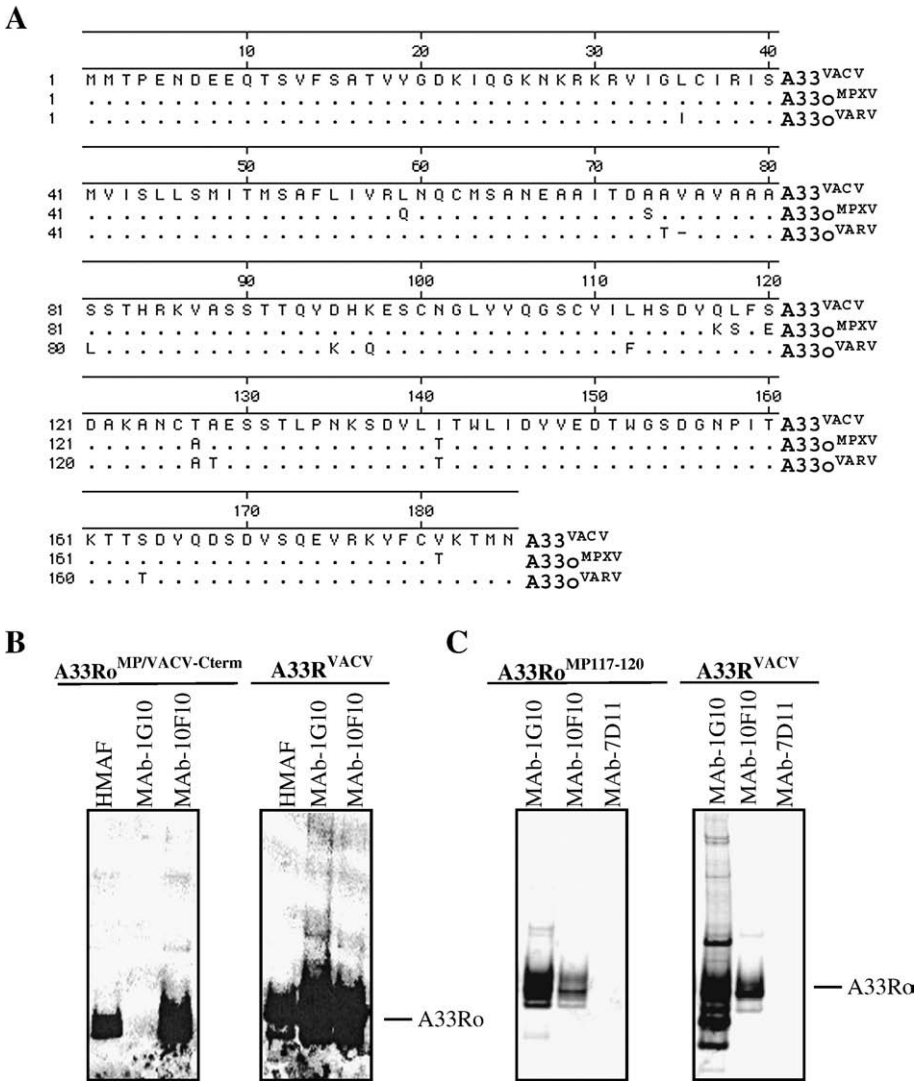

Fig. 2. Rescue of MAb-1G10 binding to A33o^{MPXV}. A. Comparison of the predicated amino acid sequences of A33^{VACV} (strain Connaught), A33o^{MPXV} (strain Zaire79) and A33o^{VARV} (Bangladesh). Amino acids 1–31 contain the predicted N-terminal cytoplasmic tail, amino acids 32–57 contain the predicted signal sequence/transmembrane, amino acids 58–185 contain the extracellular domain (Roper et al., 1996). There are also two predicted N-linked glycosylation sites at positions 125 and 135 (Roper et al., 1996). The A33 from VACV strain Connaught is identical to that of VACV strain WR. B. COS cells were transfected with pWRG/A33R or pWRG/A33Ro$^{MP\text{-}VACV\text{-}Cterm}$ and subjected to RIPA. Cell lysates were incubated with MAb-10F10, MAb-1G10 or, for a positive control, hyperimmune mouse serum. RIPA was performed as described in Fig. 1A. C. COS cells were transfected with pWRG/A33R or pWRG/A33Ro$^{MP117–120}$ and subjected to RIPA. Cell lysates were incubated with MAb-10F10, MAb-1G10, and a control antibody MAb-7D11 (anti-L1). RIPA was performed as described in Fig. 1A.

double changes (K117Q/S118L, K117Q/E120S, and S118L/E120S). To determine if these changes were sufficient to allow an interaction by MAb-1G10, COS cells were transfected with A33R^{VACV} and A33Ro^{MPXV} and the mutant constructs and analyzed by flow cytometry. As determined by the forward scatter, MAb-1G10 and MAb-10F10 both interacted with surface-expressed A33^{VACV} (Fig. 3A). MAb-10F10 also efficiently interacted with A33o^{MPXV}. Consistent with our observations of MPXV-infected cells (Fig. 1D), we observed a slight interaction between MAb-1G10 and A33o^{MPXV}. Similar to data shown in Fig. 2C, a change in all three amino acids rescued MAb-1G10 interaction (Fig. 3A). Furthermore, analysis of the A33o^{MPXV} mutants revealed that changes at position S118L and E120S and double changes at positions K117Q/S118L and S118L/E120S could also rescue MAb-1G10 interaction. No sample interacted with the control antibody, MAb-10F5. These findings suggested that amino acids 118 and 120 play a critical role in MAb-1G10 binding.

If the amino acids 118 and 120 were critical for MAb-1G10 interaction, we predicted MAb-1G10 should interact with another orthopoxvirus A33 ortholog that is homologous to VACV at these sites, such as A33o^{VARV}. Thus, the ability of MAb-1G10 to interact with A33o^{VARV} was examined by flow cytometry. COS cells were transfected with DNA-encoding A33R^{VACV} or A33Ro^{VARV}, and 48 h post-transfection, cells were incubated with MAb-10F10, MAb-1G10, or the negative control antibody, MAb-10F5. As demonstrated by the forward scatter, both MAb-10F10 and MAb-1G10 interacted with A33o^{VARV} to a degree similar to that observed with VACV^{A33} (Fig. 3B). These results supported our conclusion that amino acids 118 and 120 are involved in MAb-1G10 antibody interaction with A33o.

Cross-protective efficacy of A33Ro^{MPXV} DNA against VACV challenge

Because our findings indicated protective epitopes on the A33o molecule differ among MPXV, VARV, and VACV, we hypothesized that vaccination with A33R (or ortholog) DNA from one orthopoxvirus might not efficiently cross-protect against heterologous viral challenge. To test this hypothesis, we investigated the capacity for a DNA vaccine consisting of either A33R^{VACV} or A33Ro^{MPXV} to protect mice from lethal challenge with VACV. Mice were vaccinated with DNA encoding-A33R^{VACV}

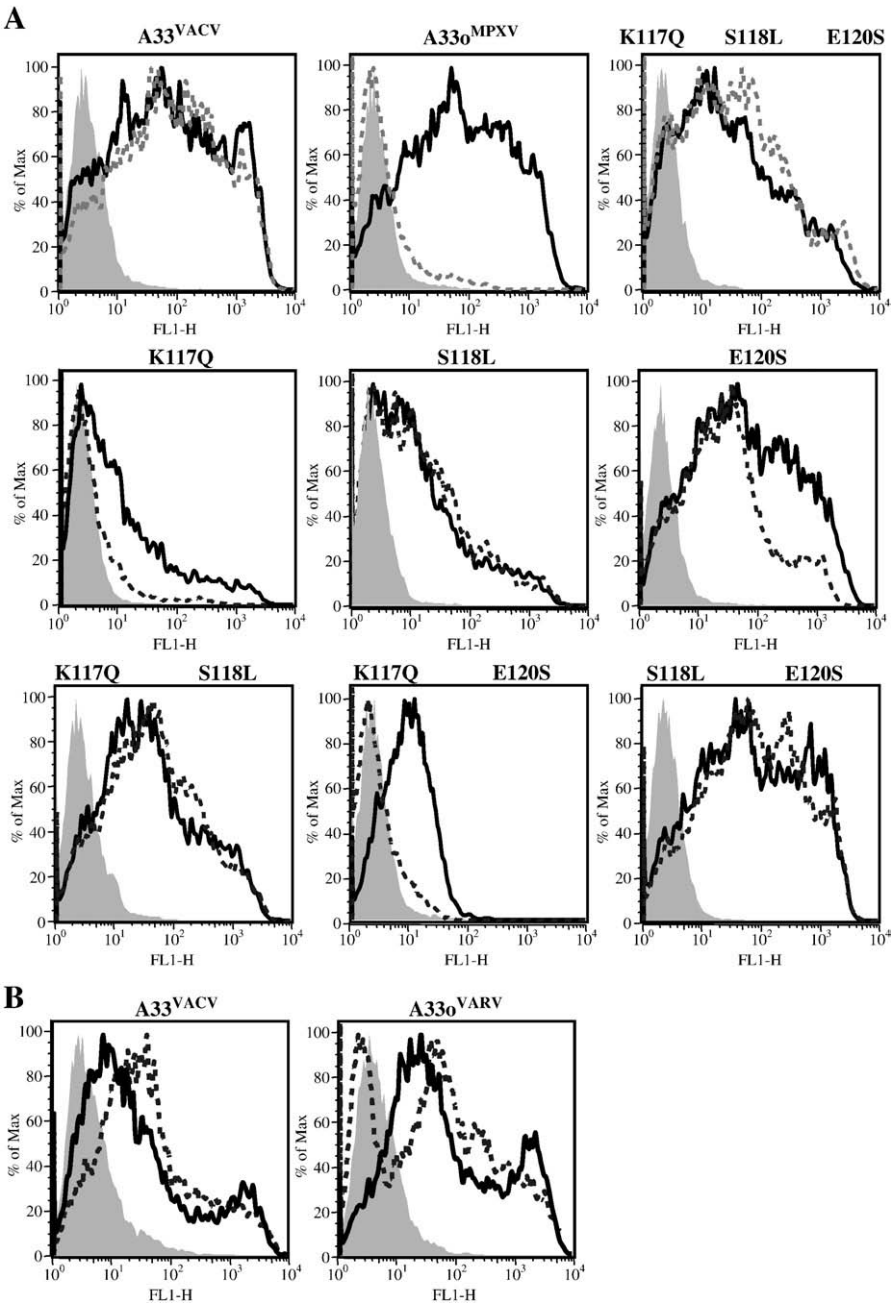

Fig. 3. Characterization of the amino acids involved in MAb-1G10 binding. A. COS cells were transfected with pWRG/A33R, pMPXV/A33Ro or the mutagenized constructs. Next, a portion of transfected COS cells (~1×10^6 cells per sample) were incubated with MAb-10F10 (solid black line), MAb-1G10 (dashed grey line) or a control antibody MAb-10F5 (solid grey area) followed by a secondary anti-mouse antibody conjugated to an Alexfluro488 fluorochrome. Antibody interactions with surface-expressed proteins were then analyzed by flow cytometry. Each analysis scanned a total of 10,000 cells/sample. B. COS cells were transfected with pWRG/A33R or pVAR/A33Ro. 48 h post-transfection, antibody interactions with surface-expressed $A33^{VACV}$ or $A33o^{VARV}$ were assessed by flow cytometry using MAb-10F10 (solid black line), MAb-1G10 (dashed grey line), or a negative control antibody MAb-10F5 (solid grey area), as described above.

or $A33Ro^{MPXV}$ three times at 3-week intervals by gold-particle bombardment using a gene-gun. Two weeks after the final vaccination, antibody responses against $A33^{VACV}$ or $A33o^{MPXV}$ were evaluated by ELISA with purified $A33^{VACV}$ or $A33o^{MPXV}$ proteins. Mice vaccinated with either $A33R^{VACV}$ or $A33Ro^{MPXV}$ elicited a similar antibody response against their respective antigen, with geometric mean titers (GMT) of 3.7 and 3.2, respectively. Three weeks after the final vaccination, $A33R^{VACV}$ and $A33Ro^{MPXV}$ vaccinated mice were challenged intranasally with 2×10^6 pfu of VACV, strain IHD-J. Control mice vaccinated three times at 3-week intervals with negative control DNA or once by tail scarification with live VACV, strain Connaught, were also included in the challenge. The percent weight loss for survivors from each group relative to the group starting weight and the percent survival for each group are shown (Fig. 4A). As expected, weights in the negative control group declined drastically starting at day two and by day seven, all mice succumbed to infection. Mice vaccinated with live virus exhibited only a moderate loss in weight that was ~10% below the starting weight at its maximum and returned to starting levels by day nine. In contrast, mice vaccinated with DNA-encoding $A33Ro^{MPXV}$ began dropping weight starting on day two. This drop continued until day 11. In this group, 40% of the mice succumbed to infection. Mice vaccinated with DNA-encoding $A33R^{VACV}$ exhibited less severe signs of disease. Weight loss for this group of mice began on day four, peaked on day five and began to increase on day six. This weight loss was never as high as the group administered with

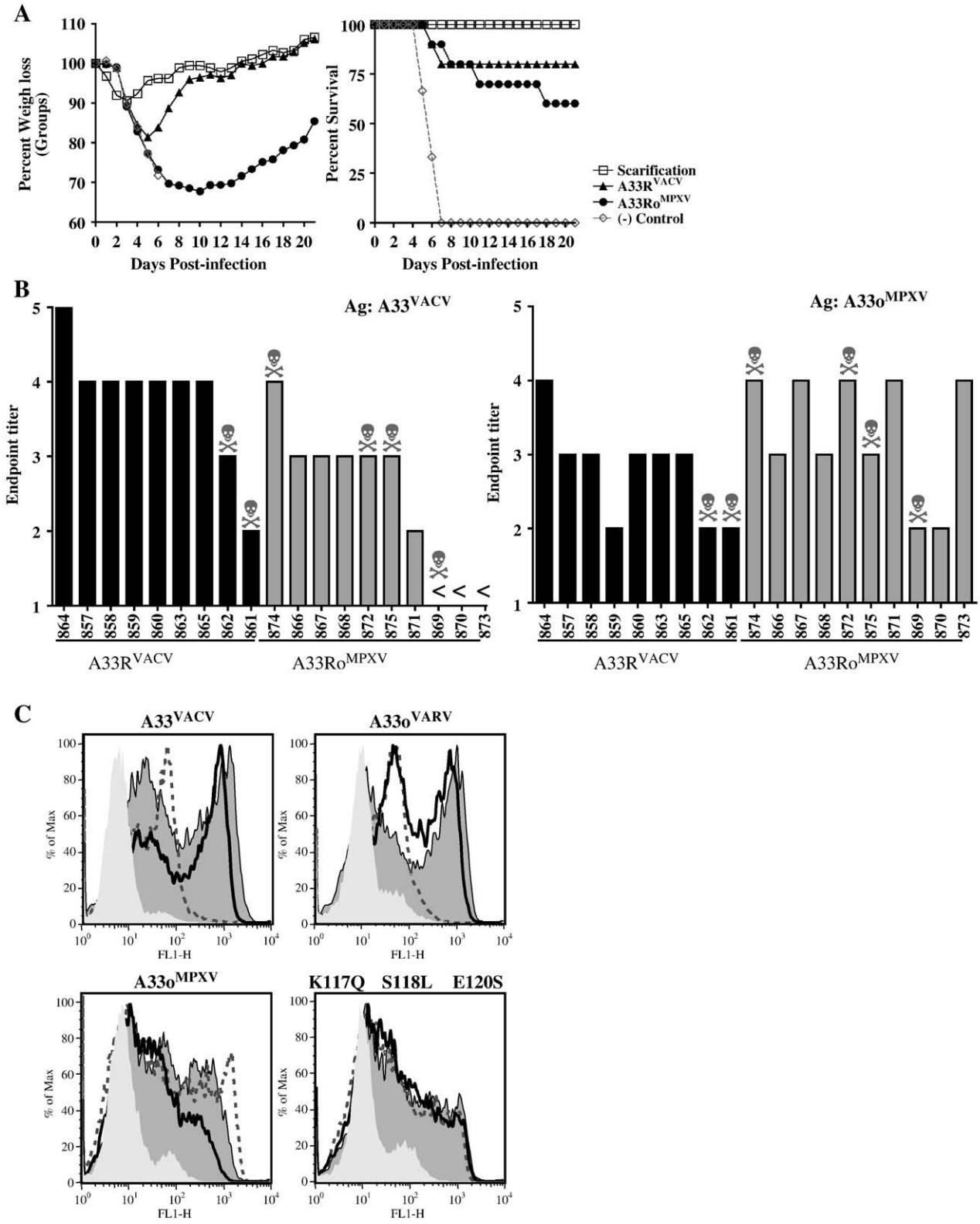

Fig. 4. Protection against heterologous viral challenge. A. Mice were vaccinated by gene-gun three times at 3-week intervals with DNA encoding either A33R^{VACV} or A33Ro^{MPXV}. Three weeks after the last vaccination, mice were challenged intranasally with 2×10^6 pfu VACV strain IHD-J. This dose represents 3 LD_{50} (Hooper et al. 2003). Mice vaccinated with live virus (VACV, strain Connaught) by tail scarification or with negative control DNA were also included in the challenge as controls. Weights of each group were monitored daily for 21 days. Data for group weights were graphed using Excel and the percent weigh loss relative to starting group weight for survivors was plotted. The percent survival was also plotted for each group. B. Antibody responses in individual mice were determined by ELISA. Sera from A33R^{VACV} (black bars) and A33Ro^{MPXV} (grey bars) vaccinated mice were serially diluted 10-fold and evaluated by ELISA with both purified A33 (left panel) or A35 (right panel). The < symbol denotes readings that were below the limits of detection. Mice succumbing to infection are denoted by the ☠ symbol. C. COS cells were transfected with pWRG/A33R, pMPXV/A33Ro, pVARV/A33Ro, or the A33Ro^{MPXV} mutant, pWRG/A33Ro$^{MP117-120}$. Antibody interactions with surface-expressed A33o were assessed by flow cytometry using MAb-10F10 (dark grey area), sera from mice vaccinated with A33R^{VACV} (black line) and A33Ro^{MPXV} DNA (dashed grey line), or sera from unvaccinated mice (light grey area).

A33Ro^{MPXV} DNA. Only 20% of the A33R^{VACV} vaccinated mice died. Additionally, weights of mice vaccinated with A33R^{VACV} returned to starting levels by day 21, whereas weights of mice vaccinated with A33Ro^{MPXV} that survived remained, on average, ~15% below their starting weight.

We performed an ELISA using sera from each vaccinated animal to determine the cross-reactivity of antibodies from mice vaccinated with A33Ro^{MPXV} or A33R^{VACV} DNA against homologous and heterologous protein (Fig. 4B). Antibodies from mice vaccinated with A33R^{VACV} did not interact with the A33o^{MPXV} antigen (GMT=2.7) as efficiently as they did with homologous VACV^{A33} antigen (GMT=3.7). Similarly, antibodies from mice vaccinated with A33Ro^{MPXV} did not interact with A33^{VACV} antigen (GMT=2.1) as efficiently as they did with homologous A33o^{MPXV} antigen (GMT=3.2). We also observed that animals vaccinated with A33R^{VACV} DNA that succumbed to challenge had the lowest antibody responses against A33^{VACV}, suggesting low titers in this group led to poor survivability. Interestingly, some A33Ro^{MPXV} vaccinated mice survived challenge yet had cross-reactive anti-A33 responses below the level of detection. In contrast, one mouse vaccinated with A33Ro^{MPXV} DNA that died from challenge had the strongest cross-reactive response. These findings indicated that there is reduced cross-reactivity of serum antibodies between A33 orthologs. Our data also indicate that the level of cross-reactivity of antibodies from A33Ro^{MPXV}-vaccinated mice with the A33^{VACV} molecule did not correlate with the survivability of animals.

We next investigated if amino acids 117, 118, and 120 played a role in the poor cross-reactivity of antibodies from mice vaccinated with A33R^{VACV} DNA with A33o^{MPXV} protein. To this end, cells were transfected with A33R^{VACV}, A33Ro^{VARV}, A33Ro^{MPXV} or the mutant A33Ro^{MPXV} that had nucleotide changes that altered amino acids at positions 117, 118, and 120 into those of A33^{VACV}. Transfected cells were then incubated with MAb-10F10 or pooled sera from unvaccinated mice and mice vaccinated with A33R^{VACV} or A33Ro^{MPXV} DNA. Antibody interactions with the various A33 orthologs were then assessed by flow cytometry. We observed an interaction between MAb-10F10 and each ortholog, demonstrating that cells were efficiently transfected with each molecule. Serum antibodies from mice vaccinated with A33Ro^{MPXV} DNA reacted efficiently with A33o^{MPXV} protein, but a marked reduction in interaction was observed with the A33^{VACV} and A33o^{VARV} molecules (Fig. 4C). Conversely, serum antibodies from A33R^{VACV} DNA-vaccinated mice reacted efficiently with A33^{VACV} and A33o^{VARV} protein, but less so with A33o^{MPXV}. However, interactions of serum antibodies from mice vaccinated with A33R^{VACV} DNA were improved if A33o^{MPXV} amino acids at positions 117, 118, and 120 were changed to those of A33^{VACV} (Fig. 4C). These data confirm the ELISA findings (Fig. 4B) and showed that mice vaccinated with DNA encoding each ortholog interacted best with their respective proteins. Furthermore, the improved interactions of anti-A33^{VACV} serum antibodies with mutant A33o^{MPXV} molecules strongly suggests that amino acids 117, 118, and 120 play a critical role in A33o antibody epitopes in mice vaccinated with A33Ro DNA.

Discussion

Amino acids involved in protective antibody binding

Several studies have found that antibodies are critical for protection against secondary orthopoxvirus infections (Edghill-Smith et al., 2005; Panchanathan et al., 2005, 2006, 2008). In responses to these findings, recent investigations have sought to identify critical antibody epitopes of several poxvirus subunit vaccine targets, including B5 and L1 (Aldaz-Carroll et al., 2005a,b; 2007; Su et al., 2007). The A33 protein is a valuable target of subunit vaccines and immunotherapeutics (Fang et al., 2006; Fogg et al., 2004; Galmiche et al., 1999; Heraud et al., 2006; Hooper et al., 2000, 2003, 2002, 2004; Lustig et al., 2005; Xiao et al., 2007). However, little is understood regarding antibody domain(s) critical for protection, or the mechanism of protection. By capitalizing on the inability of a MAb against A33^{VACV} to efficiently bind the A33o^{MPXV} ortholog, we were able to determine that at least one antibody-binding domain involves amino acids 118 and 120 (Figs. 2 and 3). This finding was based on the observation that changing the A33o^{MPXV} amino acids at either of these positions rescued MAb-1G10 binding (Figs. 2 and 3). Additionally, we showed that MAb-1G10 bound A33o^{VARV}, whose amino acids are identical to those of A33^{VACV} at these positions (Fig. 3B). Our conclusion that this region of A33 is involved in protective immunity is based on the finding that MAb-1G10 is a protective antibody capable of passively protecting animals from lethal VACV challenge (Chen et al., 2007; Lustig et al., 2005).

Our findings did not discern whether amino acids 118 and 120 directly interact with MAb-1G10 or function indirectly by impacting the conformation of the physical epitope. However, findings in the literature support the conclusion that this region constitutes a physical antibody-binding domain. Heraud et al. reported that serum antibodies from NHP vaccinated with A33Ro^{MPXV} DNA and/or protein reacted with short (15-mer) peptides homologous to regions of the A33o^{MPXV} protein that contained amino acids 117, 118, and 120 (Heraud et al., 2006). In that study, we suggested that this region is important for protective antibody interaction. However this conclusion was not based on experimental evidence and it was not determined if antibodies binding to this region were in fact protective or just part of the polyclonal antibody pool generated against A33o^{MPXV}. Using these same peptides, we found that NHPs vaccinated by gene-gun with A33Ro^{MPXV} DNA also developed anti-A33 responses targeting this region of the A33o molecule (Golden, J.W. and Hooper, J.W., unpublished observations). Recently, Chen et al. developed chimpanzee/human hybrid anti-A33 antibodies that can provide *in vivo* protection against orthopoxvirus challenges (Chen et al., 2007). The authors determined that the region involved in antibody interaction encompassed amino acids 99 and 185, which includes the amino acids identified in our study. In another study, Roper et al. observed that two anti-A33^{VACV} MAbs, MAb 4 and MAb 105, failed to interact with ectromelia virus (ECTV) (Roper et al., 1996). A33o^{ECTV} contains the same amino acids as A33o^{MPXV} at positions 117, 118, and 120, suggesting that perhaps MAb 4 and MAb 105 interact with this same region of A33. It is curious that antibodies elicited by various experimental means (NHP vaccinated with protein/DNA or with live virus and mice infected with VACV) all target this region of the A33 protein. Together these findings suggest this region of A33 may be a hotspot for protective antibody interaction. This conclusion is further supported by the fact that MAb-10F10 is also a protective antibody (Golden, J.W. and Hooper J.W., unpublished findings and Hooper et al., 2002), and competes with MAb-1G10 for binding in competition ELISAs (Hooper, J.W, and Schmaljohn, A, unpublished findings).

While MAb-1G10 did not interact with A33o^{MPXV} in the RIPA (Fig. 1A) or with purified A33o^{MPXV} by ELISA (Fig. 1B), we did observe a slight interaction with A33o^{MPXV} by flow cytometry (Fig. 3A) and with A33o^{MPXV} expressed in MPXV-infected cells (Fig. 1D). This could be explained by the interaction of A33o^{MPXV} with a viral and/or cellular membrane, which would be absent in detergent-treated protein (RIPA) or *Escherichia coli*-expressed A33 ectodomain (ELISA). In contrast, the flow cytometry experiment examined surface-expressed A33 bound to the cellular membrane (Fig. 3), and in infected cells, the molecule would be present in both cellular and viral membrane (Fig. 1D). The interaction of the A33 transmembrane region with a membrane might induce a conformational shift in the molecule making the epitope more accessible to MAb-1G10. Additionally, the fact that we observed some interaction of MAb-1G10 with A33o^{MPXV} makes it likely that more amino acids are involved in antibody binding. Our findings also indicate that the antibody epitope is likely to be conformational, as reduction of the A33^{VACV} protein disrupted the interaction of both MAb-10F10 and MAb-1G10 (Fig. 1C). A similar observation was noted by Chen et al., in that reduction of the VACV^{A33} molecule disrupted interactions of a anti-A33 chimpanzee MAb (Chen et al., 2007). Further studies, especially co-crystallization of the MAb-10F10 and MAb-1G10 FAb fragments bound

to the A33^{VACV} and A33o^{MPXV} molecule will be needed to fully map the antibody epitope(s). Such information will be a valuable guide for the development of A33-targeted immunotherapeutics and subunit vaccines that would ensure efficient cross-reactivity against orthopoxviruses.

The mechanism(s) whereby antibodies against A33 protect is not well understood. There is evidence to suggest that anti-EV antibodies block EV release from cells, but do not efficiently neutralize virus (Vanderplasschen et al., 1997). Perhaps this region of A33 possesses a functional domain required for EV release/spread that is blocked by anti-A33 antibodies. Alternatively, this region may be optimally positioned to allow bound antibodies to fix complement, leading to virus inactivation. This latter possibility is supported by findings that, at least in cell culture, anti-EV antibodies in the presence of complement can disrupt EV membranes making particles susceptible to neutralization by anti-MV antibodies (Lustig et al., 2004). Unfortunately, *in vivo* evidence for this mechanism is lacking. Clearly, more studies will be required to fully address the mechanism of protection elicited by anti-A33 antibodies.

Implications for cross-protection

In this study, we examined if differences in the A33 molecule could affect the cross-protective efficacy of a A33R-based molecular vaccine. Indeed, we observed a marked reduction in the capacity of an A33Ro^{MPXV} vaccine to cross-protect against weight loss after VACV challenge (Fig. 4). Our data suggest the poor cross-protection was associated with amino acids 117, 118, and 120, because sera from mice vaccinated with A33R^{VACV} DNA, while reacting poorly with A33o^{MPXV} protein, could interact with mutant A33Ro^{MPXV} containing changes in these amino acids to those of VACV (Fig. 4C). Curiously, there was not an absolute correlation with anti-A33o^{MPXV} cross-reactive antibody titers and survivability in mice vaccinated with A33Ro^{MPXV} DNA (Fig. 4B). These findings suggest that despite having a high cross-reactive titer, in some animals only a small portion, if any, of the polyclonal antibodies produced from vaccination cross-reacted with a protective epitope(s). Additionally, in some animals cross-reactivity with VACV^{A33} was low, yet the animals survived. This may suggest that despite having low cross-reactivity, a large enough portion of the polyclonal antibody pool could interact with protective domains in the A33 molecule to shield the animal from lethal infection. Alternatively, this result could indicate that a T-cell response is contributing to cross-protection.

Fang et al. have recently shown that mice vaccinated with A33o^{ECTV}, or A33^{VACV} protein in incomplete Freund's adjuvant (IFA) are protected from ECTV challenge (Fang et al., 2006). Similar to our findings, antibodies from vaccinated mice reacted best with homologous protein compared to the heterologous ortholog. However, mice were completely protected from ECTV challenge regardless of the A33 ortholog used for vaccination. The ECTV challenge model used by Fang et al. and the VACV challenge model used here differ not only in the virus used, but also the dose and route of challenge. Thus, it is difficult to determine why vaccination with A33 was completely protective in the ECTV model, whereas vaccination with A33Ro^{MPXV} was less protective than A33 at protecting in the VACV, strain IHD-J model. It is possible that protection of mice from ECTV challenge does not require as high of a protective anti-A33 immune response compared to that required for protection from VACV strain IHD-J, or that the anti-A33 immune response was sufficiently potent after vaccination with purified protein and adjuvant to afford complete cross-protective immunity. The IHD-J strain of VACV is known to produce large amounts of extracellular EV in cell culture (Payne, 1979), and this has been mapped to a mutation in the A34R gene (Blasco et al., 1993). If efficient production and spread of IHD-J extracellular EV occurs *in vivo*, then a very potent anti-A33 immune response might be required to protect against VACV, strain IHD-J. It is possible that heterogenicity in the A33 immunogens used to vaccinate could affect the response enough to be manifested as a difference in protective efficacy. Regardless, the results of our challenge experiment (Fig. 4) demonstrate that at least in some cases A33 does not provide complete cross-protection against heterologous challenge.

Other groups have observed poor cross-reactivity of antibodies against orthopoxvirus vaccine targets. As stated above, this includes Roper et al. who reported some anti-A33^{VACV} MAbs do not interact with A33^{ECTV} (Roper et al., 1996). Aldaz-Carroll et al. recently found that antibodies against B5^{VACV} do not cross-react efficiently with the B5o^{VARV} (Aldaz-Carroll et al., 2007). Thus, inadequacies in cross-reactivity exist for other orthopoxvirus subunit vaccine targets. To circumvent cross-protective deficiencies and otherwise bolster protective immunity, several groups, including ours, combined multiple protective orthopoxvirus immunogens into molecular smallpox vaccines. The 4pox molecular smallpox vaccine was designed to target immunogens associated with both the MV and EV forms of orthopoxviruses. For redundancy, two targets are present on the MV (L1 and A27) and two are present on the EV (A33 and B5), as well as on infected cells (Moss, 2001; Resch et al., 2007; Smith et al., 2002). The ability for this vaccine to cross-protect against MPXV challenge in NHPs has been reported (Hooper et al., 2004). Fogg et al. have also reported that a combination of three of our four VACV immunogens, VACV-L1, -B5, and -A33, can protect against MPXV challenge in NHPs when delivered as purified protein in the presence of an adjuvant (Fogg et al., 2007). It is likely that in both these NHP studies, antibodies against B5 compensated for poorly cross-reactive anti-A33o antibodies. From this, we can also hypothesize that a vaccine containing the A33o^{MPXV} might elicit a suboptimal immune response against VARV, whereas a vaccine containing the A33^{VACV} or A33o^{VARV} would elicit good anti-A33 responses against VACV and VARV, but not against MPXV. We are currently evaluating the cross-reactive immune responses generated in mice vaccinated with the 4pox genes derived from VACV, MPXV, and VARV. These data will shed light on which orthopoxvirus gene sequences should be used in a subunit vaccine to provide the broadest protection. It might also be possible to use engineered immunogens, such as A33Ro$^{MP117-120}$, to elicit higher levels of cross-protective immunity against MPXV, VACV and VARV.

Any subunit vaccine against orthopoxviruses must be designed to protect against several species of orthopoxviruses, including MPXV and VARV and also genetically modified poxviruses. In this respect, the obvious choices of targets for molecular subunit vaccines have been those that lead to the generation of protective immune responses and are also highly homologous molecules among the orthopoxvirus family. Data presented here and elsewhere (Aldaz-Carroll et al., 2007; Roper et al., 1996) highlight a caveat to this rationale and reveal that despite high overall homology, slight variations in critical epitopes can impact the cross-protective efficacy of subunit vaccine targets. Our findings caution that adequate cross-protection by any pan-orthopoxvirus subunit vaccine will require careful evaluation of cross-protective immunity. Nevertheless, redundant targeting of multiple orthopoxvirus immunogens will likely compensate for any deficiencies in cross-reactivity that may occur for individual immunogens.

Materials and methods

Cells, viruses, and monoclonal antibodies

VACV Connaught vaccine strain (derived from the New York City Board of Health strain), VACV strain IHD-J (obtained from Dr. Alan Schmaljohn), and MPXV strain Zaire79 (obtained from Dr. John Huggins) were all maintained in VERO cell (ATCC CRL-1587) monolayers grown in Eagle minimal essential medium (EMEM), containing 5% heat-inactivated fetal bovine serum (FBS) (Hyclone; Logan, UT), 1% antibiotics (100 U/ml penicillin, 100 μg/ml of streptomycin, and 50 μg/ml of gentamicin), 10 mM HEPEs (cEMEM). COS-7 (COS) cells (ATCC CRL-1651) were used for transient expression experiments and were also maintained in EMEM.

Two mouse MAbs against VACV^{A33}, MAb-10F10 and MAb-1G10, were used in these studies (Hooper et al., 2000). The murine MAb-7D11 and MAb-10F5 recognize the poxvirus MV protein L1 (Hooper et al., 2000; Wolffe et al., 1995). All MAbs were produced at USAMRIID and propagated in the USAMRIID hybridoma facility.

Radiolabeled immunoprecipitation (RIPA)

The RIPA method was described previously (Hooper et al., 2000). Briefly, COS cells were transfected with pWRG/A33R pMPXV/A33Ro or mutant A33Ro constructs as indicated using Fugene6 (Roche; Indianapolis, IN). The pWRG vector has been described elsewhere (Schmaljohn et al., 1997). By 24-h post-transfection, COS cells were labeled with ^{35}S-methionine/cystine for 4 h and lysed using 4% zwittergent buffer (4% zwittergent, 0.5 M NaCL, 1 mM EDTA and 10 mM Tris [pH 8.0]). Cell lysates were incubated as indicated with two MAbs known to interact with A33R^{VACV}, MAb-1G10 and MAb-10F10 (Hooper et al., 2000) or a control antibody MAb-7D11 that interacts with L1 (Su et al., 2007; Wolffe et al., 1995). Protein–antibody complexes were immunoprecipitated using protein A. Immunoprecipitated lysates were analyzed by SDS-PAGE on 4–12% gels. Resolved gels were dried, placed on a phosphoimager screen, and examined on a Cyclone Phosphoimager reader (Packard Instruments; Groningen, Netherlands).

Purification of orthopoxvirus proteins

Purification of A33Ro^{MPXV} produced in *E. coli* was described previously (Heraud et al., 2006). Purified A33^{VACV} was prepared in *E. coli* by methods similar to those previously reported (Heraud et al., 2006); however, a new plasmid, pET21-A33RΔTM, contained the A33R^{VACV} open reading frame with the predicted transmembrane regions (i.e., 60–181) removed. A detailed description of the plasmid construction and protein purification procedure will be published elsewhere.

Protein ELISA

Purified VACV antigens, A33 or A33o^{MPXV} (50 ng/well), were diluted in 0.1 M carbonate buffer [pH 9.6] and plated in duplicate in the wells of a high-binding 96-well plate (Corning, Corning, NY). After 24 h at 4 °C, wells were blocked with phosphate-buffered saline (PBS) containing 0.05% tween (PBS-T) and 5% milk. Hybridoma supernatant containing MAbs against A33^{VACV}, MAb-10F10, and MAb-1G10 or against VACV-L1, MAb-10F5, were diluted twofold in blocking buffer (starting at 1:100) and incubated for 1 h with the purified proteins. Plates were washed four times in PBS-T and incubated with an anti-mouse IgG conjugated to horseradish peroxidase (Sigma) (1:1000) for 1 h at 37 °C. Plates were washed again four times in PBS-T and 100 μL of 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) substrate (KPL, Gaithersburg, MD) was added to each well. Reactions were stopped by adding 100 μl of ABTS stop solution of 5% (w/v) sodium dodecylsulfate to each well. The optical density (O.D.) at 405 nm was read on a Spectramax ELISA plate reader (Molecular Devices; Sunnyvale, CA). Each reaction represents the average of two reactions ± standard deviation.

End-point antibody ELISA titers from vaccinated mice

Purified A33^{VACV} or A33o^{MPXV} (50 ng/well) diluted in 0.1 M carbonate buffer [pH 9.6], were plated in duplicate in the wells of a high-binding 96-well plate (Corning). To control for background, plates were also coated with a negative control antigen, botulinum toxin (50 ng/well) (recombinant botulinum toxin purified from *E. coli* provided by Alphavax Inc.). Plates were blocked for 1 h with PBS-T and 5% milk. The indicated antibodies or mouse sera were serially diluted tenfold (starting at 1:100) in PBS-T containing 5% milk and *E. coli* lysate. Serum dilutions were incubated with the antigens for 1 h at 37 °C. Subsequent steps were identical to those described above. End-point-titers were determined as the highest dilution with an absorbance value greater than the mean absorbance value from negative control plasmid-vaccinated animals plus three standard deviations.

Immunofluorescence of virus-infected cells

5–15 pfu of VACV, strain IHD-J, or MPXV, strain Zaire79, diluted in 100 μl of EBME were adsorbed onto confluent monolayers of COS cells on coverslips for 1 h at 37 °C. After adsorption, the inoculum was removed and a 1:1 mixture (1 ml total volume/well) of 3.0% methylcellulose (w/v) in water and 2× Eagles' basal medium with Earle's salts (EBME) supplemented to contain 5% FBS, 1% antibiotics (100 U/ml penicillin, 100 μg/ml of streptomycin, and 50 μg/ml of gentamicin) was added to each well. At 48 h post-infection, medium was removed and cells were washed twice with PBS. Cells were then fixed at room temperature with 1:1 acetone:methanol for 3 m and washed three times with PBS. After fixation, cells were blocked for 1 h in blocking buffer (PBS+5% FBS+3% goat serum). After blocking, cells were incubated with MAb-10F10 and MAb-1G10 and MAb-3D7 (anti-Haantan G2) diluted 1:100 in blocking buffer for 1 h at room temperature. Cells were then washed three times in PBS and incubated with an anti-mouse secondary antibody conjugated to Alexafluor488 (Invitrogen, Carlsbad, CA) (1:500) for 30 min at room temperature. Cells were washed three more times with PBS, once with distilled water, and mounted on glass slides using ProLong Gold anti-fade reagent containing DAPI nucleic acid stain (Invitrogen). Samples were then examined by fluorescence microscopy.

Construction of chimeric A33R plasmids

Replacement of the A33o^{MPXV} terminal threonine with the last five amino acids of A33^{VACV} was accomplished by using a 5′ A33Ro^{MPXV} primer (5′-GGGGCGGCCGCATGGTGCCGCAGCAAGCATGACAG-3′ and a 3′ primer homologous to A33Ro^{MPXV} but containing the 3′ end of A33R^{VACV} (5′ GCGCAGATCTTTAGTTCATTGTTTTAACACAAAAATACTTTCTAACTTCTTG-3′). These primers incorporated a NotI and BglII restriction site (underlined), respectively. The resultant gene product was cloned into these restriction sites in the pWRG/vector, generating pWRG/A33Ro$^{MP/VACV\text{-}Cterm}$. Site-directed mutagenesis to change amino acids 117, 118, and 120 of the A33Ro^{MPXV} gene was performed in a two-step PCR reaction. The first step consisted of two distinct PCR reactions that utilized an A33Ro^{MPXV} forward (see above) or reverse (5′-GGGAGATCTTTAGTTCATTGTTTTAACACA-3′) primer combined with a mutagenic reverse or forward primer listed in Table 1. This reaction generated two fragments of the A33Ro^{MPXV} gene, each containing the desired mutation. To generate full-length A33Ro^{MPXV} containing the desired mutations, products from the two reactions were combined and a final PCR reaction using the A33Ro^{MPXV} forward and reverse primers was performed. The forward and reverse primers also introduced NotI and BglII (underlined) sites to the 5′ and 3′ ends of the genes, respectively. All PCR reactions were performed using the Phusion polymerase (New England Biolabs, Ipswich, MA). After PCR, fragments were digested with NotI and BglII, and ligated into the pWRG/vector, generating pWRG/A33Ro$^{MP117–120}$. Sequence analysis was used to verify that the changes had been successfully incorporated into the gene. These changes are identical to those of the predicted amino acid sequence for VACV, strain Connaught, at the indicated positions.

Flow cytometry

COS cell monolayers (70–80% confluent) were transiently transfected with pWRG/A33R or pMPXV/A33Ro, or the indicated mutagenized A33Ro^{MPXV} constructs using Fugene6. Transfected cells were incubated at 37 °C for 48 h, trypsinized, and washed once with EMEM. After the wash, ~1×10^6 cells were transferred to 1.5-ml tubes. Cells

Table 1
Primers used for the site-directed mutagenesis of the A33Ro^{MPXV} gene

Mutated amino acids	Forward primer[a]	Reverse primer[a]
K117Q	ATTACATTCAGACTATCAGTCATTCGAGGATGCTAAAGCA	TGCTTTAGCATCCTCGAATGACTGATAGTCTGAATGTAAT
S118L	ATTACATTCAGACTATAAGTTATTCGAGGATGCTAAAGCA	TGCTTTAGCATCCTCGAATAACTTATAGTCTGAATGTAAT
E120S	ATTACATTCAGACTATAAGTCATTCTCGGATGCTAAAGCA	TGCTTTAGCATCCGAGAATGACTTATAGTCTGAATGTAAT
K117Q, S118L	ATTACATTCAGACTATCAGTTATTCGAGGATGCTAAAGCA	TGCTTTAGCATCCTCGAATAACTGATAGTCTGAATGTAAT
K117Q, E120S	ATTACATTCAGACTATCAGTCATTCTCGGATGCTAAAGCA	TGCTTTAGCATCCGAGAATGACTGATAGTCTGAATGTAAT
S118L E120S	ATTACATTCAGACTATAAGTTATTCTCGGATGCTAAAGCA	TGCTTTAGCATCCGAGAATAACTTATAGTCTGAATGTAAT
K117Q, S118L, E120S	ATTACATTCAGACTATCAGTTATTCTCGGATGCTAAAGCA	TGCTTTAGCATCCGAGAATAACTGATAGTCTGAATGTAAT

[a] All primers listed in 5′–3′ direction with the position of the mutation(s) underlined.

were incubated with the MAb-10F10, MAb-1G10, or MAb-10F5 (1:100) for 1 h at room temperature in FACS buffer (PBS, 5% FBS and 0.1% sodium azide). Alternatively, cells were incubated with serum from mice vaccinated with A33Ro DNA (1:50). After incubation with the primary antibody, cells were pelleted by centrifugation at 750 ×*g* for 3 m and washed twice with PBS. Cells were next incubated with anti-mouse Alexafluor488 (Invitrogen) (1:500) for 30 min at room temperature. After incubation with the secondary antibody, cells were pelleted by centrifugation at 750 ×*g* for 3 min. Washed cells were resuspended in 1 ml of FACS buffer. Flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson; San Jose, CA). Data were collected and analyzed using FlowJo software (Tree Star INC; Ashland, OR). A total of 10,000 cells were analyzed for each sample.

Western blot analysis

COS cell monolayers (70–80% confluent) were transiently transfected with pWRG/A33R, or empty vector using Fugene6. Transfected cells were incubated at 37 °C for 48 h, after which cells were collected by centrifugation at 179 ×*g*, washed twice in chilled PBS, and lysed in tris lysis buffer (10 mM Tris [pH 7.5], 2.5 mM $MgCl_2$, 100 NaCl, 0.5% Triton X-100, 5 μg/μl of leupeptin [Sigma], 1 mM PMSF). After centrifugation at 179 ×*g* to remove cellular debris, samples were resuspended in protein sample buffer (0.125 M Tris [pH 8.0], 1% SDS, 0.01% bromphenol blue, 10% sucrose) with or without the addition of 5% β-mercaptoethanol as indicated. Protein samples then were analyzed by electrophoresis on SDS-10% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, Calif.). Nitrocellulose membranes were blocked overnight at 4 °C in tris-buffered saline (10 mM Tris [pH 8.0], 150 mM NaCl and 0.05% Tween) (TBS-T) containing 5% nonfat dry milk, rinsed with TBS-T, and incubated with MAb-10F10 or MAb-1G10 (1:1000) for 1 h. Membranes were subsequently washed with TBS-T and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse immunogloblin G (IgG) (1:10000 in TBS-T) (Amersham, Arlington Heights, Ill.). Bound antibody was detected by treating the nitrocellulose filters with enhanced chemiluminescence (ECL) detection reagents (Amersham) and exposing the filters to film.

DNA vaccination with gene-gun

The DNA vaccination procedure was described previously (Hooper et al., 2000; Schmaljohn et al., 1997). Briefly, plasmid DNA pWRG/A33Ro, pMPXV/A33Ro, or pWRG/HTN G1 was precipitated onto ~2-μM diameter gold beads at a concentration of 1 μg of DNA/1 mg of gold. DNA-gold mixtures were coated on the inner surface of irradiated Tefzel tubing and the tubing was cut into 0.5-in cartridges. Each cartridge contained ~0.25–0.5 μg of DNA coated on 0.5 mg of gold. All cartridges were quality controlled to ensure the presence of DNA. For vaccinations, the abdominal fur of BALB/c mice was shaved and DNA-coated gold was administered using a gene-gun (Powdermed delivery device, Powdermed, INC; Oxford, England) and compressed helium gas at 400 p.s.i. Two cartridges were used per mouse at each vaccination. Mice were vaccinated three times at 3-week intervals. All mice were at least 7–9-weeks old at the start of vaccination.

Scarification

Scarification was performed by placing a 10-μl drop of PBS containing 8×10^6 10^6 pfu of VACV, strain Connaught, near the base of the tail on each mouse. The tail was then scratched ~15–20 times using a needle on a tuberculin syringe.

Viral challenges

Mice were anesthetized and weighed before intranasal administration with a plastic pipette tip containing 50 μl of PBS with 2×10^6 pfu of VACV, strain IHD-J. This dose represents at least three times the LD_{50}. Mice were observed and weighed daily for 21 days post-infection. Moribund mice (>30% body weight) were euthanized.

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Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army or the Department of Defense.

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